

A Novel Chromogranin-A Promoter-Driven Oncolytic Adenovirus for Midgut Carcinoid Therapy

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Abstract Purpose: The use of replication-selective oncolytic adenoviruses is an emerging therapeutic approach for cancer, which thus far has not been employed for carcinoids. We therefore constructed Ad[CgA-E1A], a novel replication-selective oncolytic adenovirus, where the chromogranin A (CgA) promoter controls expression of the adenoviral *E1A* gene.

Experimental Design: The Ad[CgA-E1A] virus was evaluated for E1A protein expression, replication ability, and cytolytic activity in various cell lines. It was also evaluated for treatment of xenografted human carcinoid tumors in nude mice. To use Ad[CgA-E1A] for the treatment of carcinoid liver metastases, it is important that normal hepatocytes do not support virus replication to minimize hepatotoxicity. We therefore evaluated CgA protein expression in normal hepatocytes. We also evaluated CgA gene expression in normal hepatocytes and microdissected tumor cells from carcinoid metastases.

Results: We found that Ad[CgA-E1A] replicates similarly to wild-type virus in tumor cells with neuroendocrine features, including the BON carcinoid cell line and the SH-SY-5Y neuroblastoma cell lines, whereas it is attenuated in other cell types. Thus, cells where the CgA promoter is active are selectively killed. We also found that Ad[CgA-E1A] is able to suppress fast-growing human BON carcinoid tumors in nude mice. Furthermore, CgA is highly expressed in microdissected cells from carcinoid metastases, whereas it is not expressed in normal hepatocytes.

Conclusion: Ad[CgA-E1A] is an interesting agent for the treatment of carcinoid liver metastases in conjunction with standard therapy for these malignancies.

Neuroendocrine tumors of the gastroenteropancreatic tract represent a rare and heterogeneous group of tumors. They are subdivided according to the recent WHO classification into well-differentiated tumors, well-differentiated endocrine carcinomas, and poorly differentiated carcinoids. According to the old classification, they could also be classified into foregut, midgut, and hindgut tumors, according to their embryologic origin (1, 2). Classic midgut carcinoids arise from enterochromaffin cells in the mucosa of jejunum, ileum, cecum, and ascending colon, and they produce various peptides and hormones (3). Most patients with midgut carcinoids have developed metastatic disease at the time of diagnosis, with metastases most frequently detected in mesentery and liver.

Surgery is often used to control metastatic disease (4). Cytotoxic agents, biological therapies, and tumor-targeted radionuclides may prolong survival. However, curative treatments are not available, and metastatic midgut carcinoids represent a therapeutic challenge.

The use of oncolytic replication-selective adenoviruses is an emerging therapeutic approach for cancer (5), which may also be applied for carcinoids. This approach exploits the lytic property of viruses to kill tumor cells and is not dependent on transduction specificity, but on the specificity of viral replication in tumor cells. Hence, by replacing the endogenous viral promoter sequence driving the expression of early genes, such as E1A, with a tumor- or tissue-specific promoter, the virus will become a conditional replication-competent adenovirus (CRAd) that selectively replicates in certain tumors or tissues. This approach has been successfully used to construct CRAds against prostate cancer (6–8), breast cancer (9), liver cancer (10, 11), melanoma (12), lung cancer (13), colon cancer (14), neuroblastoma (15), gallbladder cancer (16), and cancer in general (17–19). CRAds have a potential advantage over replication-defective adenoviral vectors because every infected tumor cell serves as a virus-producing cell, thereby increasing virus spread and enhancing the therapeutic effect.

Chromogranin A (CgA) is an acidic hydrophilic protein of 48 kDa originally identified in chromaffin granules of the adrenal medulla. In addition to the adrenal gland, CgA is expressed by a variety of cells in endocrine, neuronal, and neuroendocrine tissues (20). Furthermore, CgA is highly

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expressed by various tumors, including carcinoid tumors, neuroblastoma, pheochromocytoma, medullary thyroid tumors, pancreatic islet cell tumors, small cell lung carcinoma, and prostate cancer. Serum CgA has proven to be a sensitive marker for the diagnosis of various types of neuroendocrine tumors (21). The promoter sequence that controls CgA gene expression contains both positive and negative control domains that ensures neuroendocrine cell type-specific pattern of transcription. Two 5' regions of the transcription start site have been identified and described: a proximal regulatory region between -92 and -20 and a distal regulatory region located between -726 and -455. Within the proximal regulatory region domain, response elements for early growth response protein 1, specificity protein 1, and cyclic AMP-response element binding protein (CREB) have been described upstream of the consensus TATA box (22-24). Within the distal regulatory region domain, the region responsible for the transcriptional effect was found between -576 and -550 (25). It contains an activator protein 1 motif and has been described as a distal regulatory element (DRE). It has been shown that the DRE by itself was substantially more effective at enhancing transcription than the entire 271-bp distal regulatory region, which suggests the presence of negative elements within the distal regulatory region fragment. It has also been shown that mutation in the activator protein 1 motif abolished the enhancing effects of the DRE on transcription. Studies indicate that transcriptional enhancement of CgA by the DRE is dependent on a unique neuroendocrine-specific DRE-binding factor, which specifically and directionally binds the DRE to assemble and synergize a functional transcriptional complex (26).

In this paper, we describe the construction of a novel oncolytic adenovirus for midgut carcinoids where the CgA promoter controls the expression of the adenoviral E1A gene, Ad[CgA-E1A]. The novel oncolytic adenovirus replicates similarly to wild-type virus in tumor cells with neuroendocrine features, whereas it is attenuated in other cell types. Furthermore, it is able to suppress carcinoid tumor growth in an experimental mouse model.

Materials and Methods

Cell lines. The human endocrine pancreatic tumor cell line BON was cultured in DMEM with Glutamax-I and F12 K Nutrient Mixture (Kaighn's Modification) at a 1:1 ratio, supplemented with 10% fetal bovine serum (FBS), 1 mmol/L sodium pyruvate and 1% penicillin-streptomycin (PEST). The human neuroblastoma cell lines SH-SY-5Y and SK-N-DZ, the breast adenocarcinoma cell line T47D, the human bladder carcinoma cell line T24, the human foreskin fibroblast cell line 1064SK, the transformed human embryonic retina cell line 911, and the transformed human embryonic kidney cell line 293T were cultured in DMEM, supplemented with 10% FBS, 1 mmol/L sodium pyruvate, and 1% PEST. The human melanoma cell lines 397mel and 526mel were cultured in Iscove's modified Dulbecco's medium supplemented with 10% FBS, 3.2 mmol/L L-glutamine, and 1% PEST. The transformed human liver cell line THLE-2 was cultured in Bronchial Epithelial Medium kit (Clonetics Corporation, Walkersville, MD), according to the American Type Culture Collection's recommendation, supplemented with 10% FBS and 1% PEST. All cell culture reagents were from Invitrogen (Carlsbad, CA) except when stated differently.

Recombinant adenoviruses. The adenovirus serotype 5 (Ad5) backbone vector pAdEasy(E3) and the Ad5 transfer vector pShuttle-i/PPT-

E1A have been described earlier.⁴ pAdEasy(E3) is a derivative of pAdEasy1 (27) that contains the full-length adenoviral E3 region.⁴ pShuttle-i/PPT-E1A is a derivative of pShuttle (27) that contains the adenoviral serotype 2 (Ad2) E1A coding sequence under transcriptional control of the 1.4-kb recombinant human PPT promoter, which is shielded from transcriptional interference from adenoviral sequences by the 1.6-kb mouse H19 core insulator.⁴

The 788-bp human CgA promoter was PCR amplified from human genomic DNA (Roche, Indianapolis, IN) using Expand high fidelity enzyme mix (Roche) and the following primers: CgA promoter forward 5'-GCG GCC GCT TCC TGG TGA AAG TGA GCC C-3' and CgA promoter reverse 5'-AAG CTT TCG GTC GAT CCT CCC GCA AG-3'. The PCR product was T/A cloned into pCR2.1 (Invitrogen) and DNA sequenced. pShuttle-i/CgA-E1A was constructed by replacing the PPT promoter with the CgA promoter. Ad[CgA-E1A] was obtained through homologous recombination of pShuttle-i/CgA-E1A and pAdEasy(E3) using BJ5183 bacterial cells. The construction of Ad[CMV-E1A] and Ad[Mock] have been described earlier (6). Recombinant adenoviruses were produced in 911 cells, purified by CsCl banding and dialyzed against a buffer containing 10 mmol/L Tris-HCl (pH, 8.0), 2 mmol/L MgCl₂, and 4% sucrose. Virus titers were determined by a fluorescence-forming unit (FFU) assay as described earlier.⁴ Viruses were stored in aliquots at -80°C.

The number of Ad[CgA-E1A] DNA copies versus the number of wild-type Ad5 DNA copies (contaminants) was evaluated by real-time PCR using the following primers: Fwd-200 5'-TAT ATA AGC GGG GCG CGA G-3' (in CgA promoter), Fwd-100 5'-CGT TCC GGG TCA AAG TTG G-3' (in E1A promoter sequence), and Rev-100 5'-GGA GGA GAA AAC TCT ACT CG-3' (in 5'UTR E1A sequence). Fwd-200 and Rev-100 were combined for Ad[CgA-E1A]-specific amplification, and Fwd-100 and Rev-100 were combined for wild-type Ad5-specific amplification.

Western blot. Cells cultured on six-well plates were transfected at 60% to 80% confluency with Ad[CgA-E1A] and Ad[CMV-E1A] at a multiplicity of infection (MOI) of 10 FFU/cell. Cells were harvested 48 h later, and total protein extracts were prepared as described earlier (6). Protein concentrations were determined using the Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL). Protein samples (40 µg) were then resolved by 10% SDS-PAGE and transferred to 0.45-µm nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were incubated with a mouse monoclonal anti-E1A antibody (M73, Neomarkers Inc., Fremont, CA), washed, and incubated with a peroxidase-conjugated anti-mouse/rabbit mix (Roche). After being washed, the blots were visualized by using the BM Chemiluminescence Western Blotting kit (Roche). The membranes were then incubated with a goat polyclonal anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA), followed by a horseradish peroxidase-conjugated donkey anti-goat antibody (Santa Cruz Biotechnology). The blots were visualized by using the Western Blotting Chemiluminescence Luminol Reagent (Santa Cruz Biotechnology).

In vitro viral replication assay. Cells cultured on six-well plates were transfected at 60% to 80% confluency with Ad[CgA-E1A], Ad[CMV-E1A], and Ad[Mock] at a MOI of 5 FFU/cell. Cells were harvested 4 and 72 h after transduction, and cell lysates were prepared by four cycles of freezing and thawing. Virus titers were determined by FFU assay as described earlier.⁴

Cell viability assay. Cells were transfected in suspension with Ad[CgA-E1A], Ad[CMV-E1A], and Ad[Mock] at a MOI of 10 FFU/cell. Two hours after transduction, the cells were plated in 96-well plates, 1 × 10⁴ cells per well. Cell viability was analyzed 3, 5, and 7 days after transduction using the MTS cell titer 96 aqueous one solution cell proliferation assay (Promega, Madison, WI) according to the manufacturer's instructions.

⁴ A. Danielsson et al. Increased therapeutic efficacy of the prostate-specific oncolytic adenovirus Ad[i/PPT-E1A] by introduction of the entire E3 region or the adenovirus death protein. 2007, submitted for publication.

Construction of a luc/GFP-expressing carcinoid cell line (BON). A firefly luciferase (luc) and humanized enhanced green fluorescent protein (EGFP)-expressing carcinoid cell line, BON(luc/GFP), was produced using the pBMN-LUC-I-GFP retroviral vector, which has been described earlier.⁴ BON cells (2×10^5 cells per well in six-well plates) were infected through 90 min of spinoculation with 3 mL of pBMN-LUC-I-GFP retroviral supernatant together with 4 $\mu\text{g}/\text{mL}$ of polybrene (Sigma-Aldrich, St. Louis, MO). After 48 h, the EGFP-positive cell fraction was sorted to a purity of 99% by using a FACSVantage DiVa Turbo Sorter (BD Biosciences, Stockholm, Sweden).

Animal studies. BON(luc/GFP) cells, assessed by flow cytometry to be >95% EGFP positive, were mixed 1:1 with Matrigel (BD Biosciences), and 5×10^6 cells were injected s.c. in the hind flank of male C57BL/6 *nu/nu* mice, 6 to 8 weeks of age (B&K Universal, Sollentuna, Sweden). The mice were then treated with intratumoral injections of Ad[CgA-E1A], 5×10^8 FFU in 40 μL 10, 12, 14, and 17 days after tumor cell injection. The control group was instead given 40 μL of PBS intratumorally. Tumor growth was followed by *in vivo* bioluminescence imaging using the IVIS-100 Imaging System (Xenogen Corporation, Alameda, CA). The mice were anesthetized using 2.5% isoflurane (Abbott Scandinavia AB, Solna, Sweden), and D-luciferin (Xenogen) resolved in sterile water (90 mg/kg) was injected i.p. The mice were then placed in a dark box with a 37°C-heated surface, and isoflurane was lowered to 1.5%. Images were taken 10 min after administration of D-luciferin. Automatic contour regions of interest were created, and the amount of viable tumor cells was quantified as photons per second per square centimeter per steradian. Tumor growth was also followed by caliper measurements, and tumor volumes were calculated as ellipsoid bodies. Tumors from Ad[CgA-E1A]-treated mice were excised 62 days after tumor cell injection, i.e., 44 days after the last Ad[CgA-E1A] treatment, and frozen in liquid nitrogen to investigate the presence of replicating viruses. The Local Animal Ethical Committee approved the experiment (ref. no. C67/4).

Statistical analysis. Statistical comparison was done using GraphPad Prism program (GraphPad Software, San Diego, CA). The Mann-Whitney *U* test was used to determine the difference in tumor sizes, and the Kaplan-Meier log-rank test was used for comparison of survival curves.

Isolation of human hepatocytes. Wedge biopsies were taken from patients who underwent liver surgery for secondary malignancy (mostly colorectal metastases). Samples were obtained from the nontumoral margin. Informed consent was obtained from each patient, and all experimental procedures were carried out in compliance with Swedish law and regulations and approved by the regional Ethical Committee at the Uppsala University Hospital (ref. no. 2004:M-223). Human hepatocytes were isolated by a perfusion technique (28). Briefly, the specimen was initially rinsed in saline solution and transported in saline at 4°C. Cold ischemic time was 50–90 min. Two large veins were identified at the cut surface, and smaller visible vessels were sutured to prevent leakage, and the Glisson's capsule was restored with tissue glue. The perfusion was done through the existing vasculature at 37°C. The first washing buffer consisted of Ca^{2+} - and Mg^{2+} -free HBSS with 0.5 mmol/L EGTA and 50 mmol/L HEPES. The perfusion lasted for 20 min without recirculation and a flow rate of 20 mL/min per cannula. A short interperfusion (2 min) was done with HBSS to rinse the specimen from EGTA. The perfusion was continued using HBSS containing 0.05% collagenase type IV and 5 mmol/L CaCl_2 . The perfusion was stopped when the liver sample was determined to be soft. After the perfusion was completed, the capsule was opened, and the hepatocytes were dissociated by gently chopping the tissue with a pair of scissors. This procedure was done in the warmed collagenase solution. The cell suspension was filtered through a 100- μm mesh into ice-cold William's medium E and then purified by three centrifugations in ice-cold William's medium E (3 min, 60 \times g, 4°C). The hepatocyte suspension was finally enriched through a Percoll gradient. Freshly isolated hepatocytes were cultured on collagen-coated six-well plates (1×10^6 viable cells per well) in William's medium E supplemented

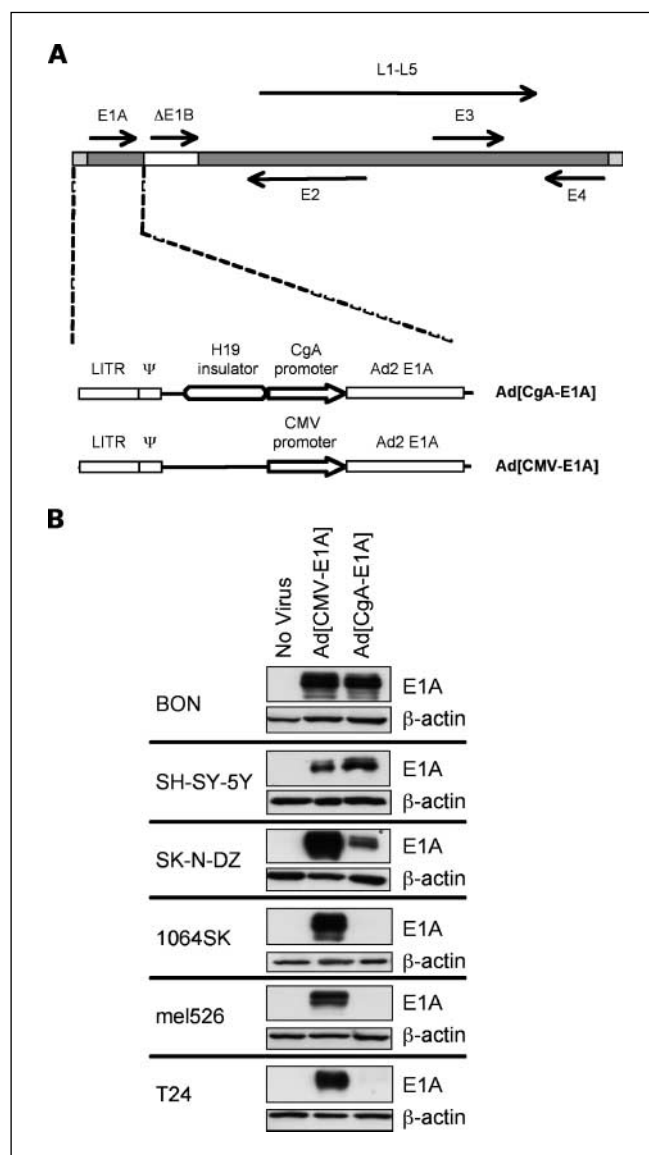


Fig. 1. Neuroendocrine cells support E1A expression after Ad[CgA-E1A] transduction. **A**, schematic illustration of recombinant adenoviruses. Ad[CgA-E1A] is a E1B-deleted human Ad5 virus where the human CgA promoter controls Ad2 E1A gene expression and where the CgA-E1A expression cassette is shielded from interfering adenoviral sequences by the mouse H19 core insulator. Ad[CMV-E1A] is a E1B/E3-deleted human Ad5 virus where the human immediate early CMV promoter controls Ad2 E1A. **B**, neuroendocrine (BON, SH-SY-5Y, SK-N-DZ) and non-neuroendocrine (1064SK, mel526, T24) cells were transduced with Ad[CgA-E1A] and Ad[CMV-E1A]. Total protein lysates were prepared after 48 h, and 40 μg samples were resolved by SDS-PAGE. E1A was detected by Western blotting using anti-E1A antibody. Anti- β -actin antibody was used as a control of protein loading.

with 10% FBS, 10 $\mu\text{g}/\text{mL}$ gentamicin, 25 IE/L insulin, 2 mmol/L L-glutamine, 0.1 $\mu\text{mol}/\text{L}$ dexamethasone and 1% PEST.

Laser-assisted microdissection and total RNA extraction. Snap-frozen midgut carcinoid mesentery and liver metastases from four individual patients were cut into 8- μm sections by using a microtome cryostat (Microm, Walldorf, Germany) and adhered to polyethylene-naphthalate membrane slides (Carl Zeiss AB, Stockholm, Sweden). Permission to collect tumor specimens was approved by the regional Ethical Committee at the Uppsala University Hospital (ref. no. Ups 02-077). Midgut carcinoid tumor cells (about 5,000 cells from each metastases) were isolated by using the PALM Robot Microbeam laser microdissection

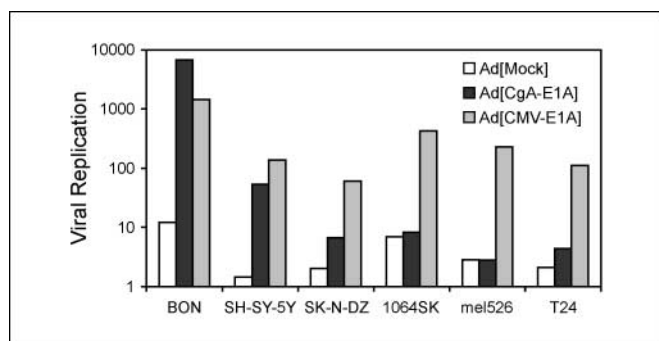


Fig. 2. Selective replication of Ad[CgA-E1A] in cells of neuroendocrine origin. A, neuroendocrine (BON, SH-SY-5Y, SK-N-DZ) and non-neuroendocrine (1064SK, mel526, T24) cells were transduced with Ad[CgA-E1A], Ad[CMV-E1A], and Ad[Mock]. Cell lysates were prepared after 4 and 72 h by four cycles of freezing/thawing. Virus titers were determined by FFU assay on 911 cells using serial dilutions of the lysates. Virus replication was calculated by dividing the viral titers at 72 h with the titers at 4 h.

system (PALM Microlaser Technologies AG, Bernried, Germany) according to the procedure described by Mücke et al. (29). Total RNA was extracted from microdissected tumor cells by using the Pico Pure RNA isolation kit (Arcturus, Mountain View, CA) according to the manufacturer's protocol. RNA was verified by using the RNA 6000 pico kit (Agilent Technologies, Palo Alto, CA) and the Agilent 2100 Bioanalyzer (Agilent Technologies).

Immunofluorescence microscopy analysis. Isolated normal hepatocytes and BON cells were plated on microscope glass coverslips placed in six-well plates. They were washed, fixed, permeabilized, and blocked for unspecific binding as described earlier (30). Cells were then incubated with a primary rabbit anti-human CgA antibody (DAKO, Copenhagen, Denmark) diluted 1:50 in PBS containing 5% calf serum. Cells were washed thrice with PBS and incubated with a secondary TRITC-conjugated anti-rabbit antibody (DAKO) diluted 1:100 in PBS containing 5% calf serum. Cell nuclei were stained with 4'-6-diamidino-2-phenylindole (DAPI; Sigma). The coverslips were washed with distilled water and mounted by using Fluoromont G (Immunokemi, Stockholm, Sweden). The samples were analyzed using a Zeiss 510 META instrument with an Axiovert 200 microscope stand (Carl Zeiss AB). All images were acquired using 63 \times magnification. Images were acquired in multitracking mode and presented as single projections.

Quantitative real-time PCR analysis. Total RNA was isolated from microdissected midgut carcinoid cells from liver metastases as described above and from normal hepatocytes, BON, and THLE-2 cells using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. About 2 ng of each RNA sample were subjected to cDNA synthesis using Superscript II RNase H⁻ Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. Primers for quantitative real-time PCR are CgA forward 5'-CCC CAC TGT AGT GCT GAA CC-3'; CgA reverse 5'-GGA GTG CTC CTG TTC TCC C-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward 5'-CCC ATG TTC GTC ATG GGT GT-3'; and GAPDH reverse 5'-TGG TCA TGA GTC CTT CCA CGA TA-3'. Gene-specific PCR products were measured by iCycler IQ real-time detection system (Bio-Rad) using iQ SYBR Green supermix (Bio-Rad). The data were evaluated using the $2^{-\Delta\Delta CT}$ method (30, 31) using the mRNA level of GAPDH from each individual sample for normalization. Average expression with SD from triplicate samples was plotted in relation to average expression of GAPDH (set to 1).

Furthermore, BON(luc/GFP) tumors excised from Ad[CgA-E1A]-treated nude mice were sectioned (20 μ m) using a cryostat, and total RNA was isolated (RNeasy, Qiagen). About 2 μ g of RNA was used for each cDNA synthesis (Superscript II, Invitrogen). Quantitative real-time PCR was done in the iCycler IQ (Bio-Rad) using iQ SYBR Green supermix and the following primers: E1A forward 5'-ATG GGC AGT

CGG TGA TGA AGT-3' and E1A reverse 5'-CTC AGG CTC AGG TTC AGA C-3' and GAPDH primers as described above. Average expression with SD from triplicate samples was evaluated using the $2^{-\Delta\Delta CT}$ method and plotted in relation to average expression of GAPDH (set to 1).

Results

Ad[CgA-E1A] specifically replicates in and kills cells of neuroendocrine origin. The constructed Ad[CgA-E1A] virus is a E1B-deleted human Ad5 virus where the human CgA promoter controls Ad2 E1A gene expression and where the CgA-E1A expression cassette is shielded from interfering adenoviral sequences by the mouse H19 core insulator (Fig. 1A). The Ad[CMV-E1A] control virus is a E1B/E3-deleted Ad5 virus where the human immediate early CMV promoter controls Ad2 E1A gene expression (Fig. 1A). The Ad[CgA-E1A] virus batch was examined for wild-type Ad5 contaminants by real-time PCR. We verified that there was <1 copy of wild-type Ad5 per 10⁶ Ad[CgA-E1A] vector copies.

To investigate promoter specificity, cells of neuroendocrine or non-neuroendocrine origin were transduced with the Ad[CgA-E1A] virus. We found that the human pancreatic carcinoid cell line BON and the human neuroblastoma cell line SH-SY-5Y supported E1A protein expression from Ad[CgA-E1A] as efficiently as from Ad[CMV-E1A] (Fig. 1B). The human neuroblastoma cell line SK-N-DZ also supported E1A protein expression, although at a somewhat lower level than Ad[CMV-E1A]. In sharp contrast, E1A protein expression was not detected in Ad[CgA-E1A]-transduced human foreskin

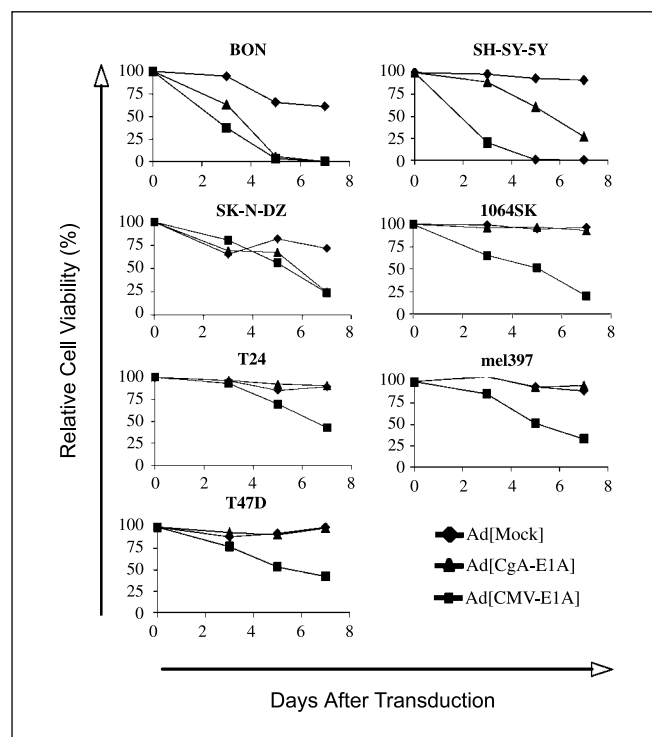


Fig. 3. Specific cytotoxicity of Ad[CgA-E1A] on cells of neuroendocrine origin. Cells of various origin were transduced with Ad[CgA-E1A], Ad[CMV-E1A], and Ad[Mock]. Cell viability was examined on days 3, 5, and 7 by MTS assay and expressed in relation to the viability of nontransduced cells. Average enzymatic activities from triplicate samples are shown.

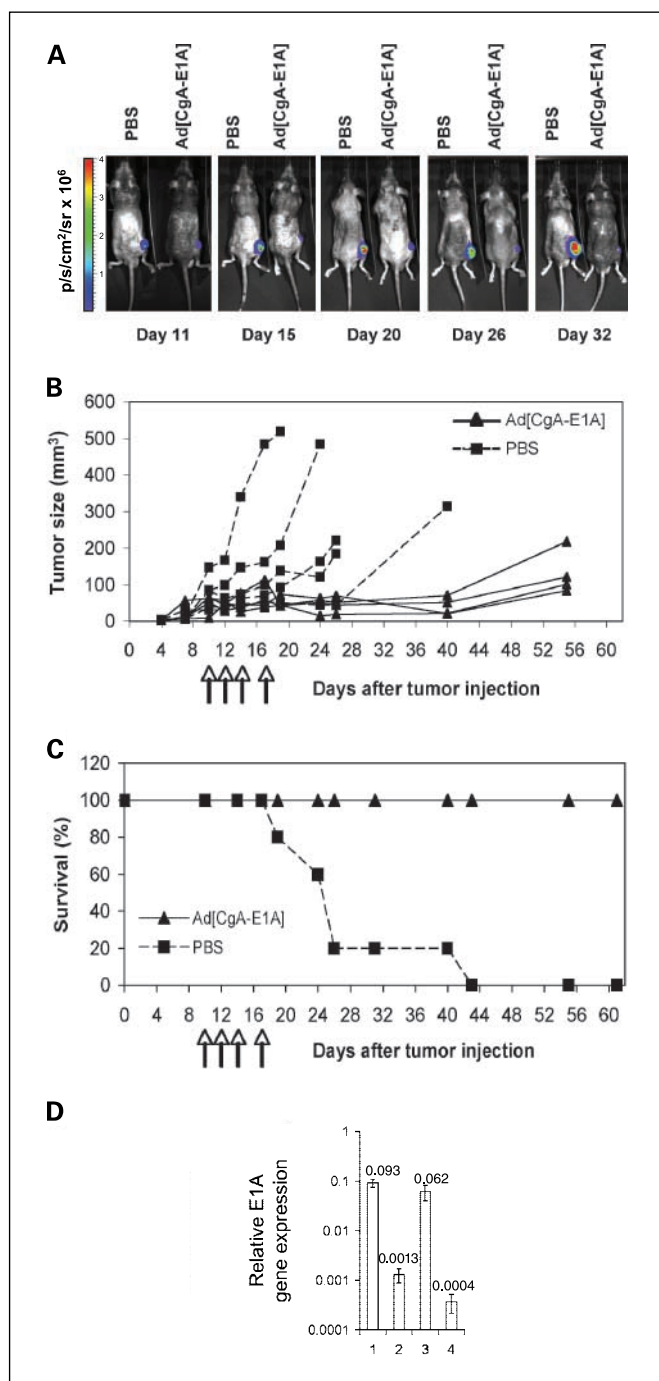


Fig. 4. Ad[CgA-E1A] suppresses carcinoid tumor growth *in vivo*. BON(luc/GFP) cells were injected s.c. in nude mice. After 10 d, the established tumors were treated with intratumoral injections of Ad[CgA-E1A] or PBS. The tumors were treated again on days 12, 14, and 17. Tumor growth was followed in the *in vivo* bioluminescence imaging system (IVIS) and through caliper measurements of tumor sizes. **A**, BON(luc/GFP) tumor growth in two mice, one mouse treated with Ad[CgA-E1A] and the other treated with PBS. On days indicated below images, the mice were anesthetized and injected with D-luciferin, and images were collected. **B**, tumor sizes for individual Ad[CgA-E1A]-treated mice (\blacktriangle and ---) and PBS-treated mice (\blacksquare and - - -) are plotted. Arrows, time points of treatment. **C**, survival curves for Ad[CgA-E1A]-treated mice (\blacktriangle and ---) and PBS-treated mice (\blacksquare and - - -). Arrows, time points of treatment. **D**, four individual tumors from Ad[CgA-E1A]-treated mice were excised after 61 d. RNA was isolated, cDNA was synthesized, and quantitative real-time PCR was done with E1A-specific primers. C_T values were calculated, and the data were evaluated using the $2^{-\Delta\Delta C_T}$ method with GAPDH from each individual sample for normalization. Columns, average values from triplicate samples; bars, SD.

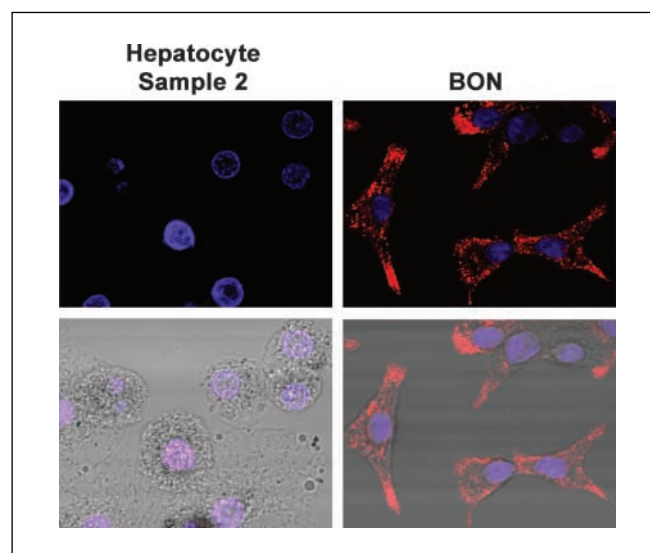


Fig. 5. Freshly isolated hepatocytes do not express CgA. Normal hepatocytes were isolated from liver surgery material through a two-step collagenase perfusion technique. Freshly isolated hepatocytes (from individual 2) were plated on coverslips overnight, washed, and incubated with a TRITC-labeled anti-CgA antibody and DAPI. BON was used as a positive control. CgA expression (red) and DAPI staining (blue) were evaluated by immunofluorescence microscopy.

fibroblast 1064SK, human melanoma 526mel, or human bladder carcinoma T24 cells, showing that the CgA promoter is not active in these non-neuroendocrine cell lines. As expected, E1A protein expression was detected in all cell lines transduced with Ad[CMV-E1A] (Fig. 1B).

Viruses were then evaluated for replication ability in the same panel of cell lines. Figure 2 shows that Ad[CgA-E1A] replication is very efficient in BON cells, with a replication index of 6,800, i.e., every virus gives rise to 6,800 progeny viruses within 72 h. Ad[CgA-E1A] also replicates efficiently in SH-SY-5Y, similarly to Ad[CMV-E1A] replication, whereas the replication was less efficient than Ad[CMV-E1A] in SK-N-DZ (Fig. 2). The lower replication in SK-N-DZ cells is consistent with the lower expression level of E1A protein found in Ad[CgA-E1A]-transduced SK-N-DZ (Fig. 1). In contrast, Ad[CgA-E1A] did not replicate in 1064SK, 526mel, or T24, confirming the specificity of Ad[CgA-E1A] to neuroendocrine cells. Ad[CMV-E1A] replication is efficient in all cell lines examined, whereas replication was not detected for Ad[Mock] (Fig. 2). The T24 cell line has, in some studies, been reported to be coxsackie/adenovirus receptor (CAR) negative and, therefore, a poor target for viral replication (32). However, Loskog et al. have previously reported that the T24 cell line cultured in our laboratory is CAR positive by reverse transcription-PCR (33) and Western blot.⁵ Furthermore, Loskog et al. also reported that T24 cells transduced with an adenoviral vector express the transgene (34).

The cell-killing efficacy of the Ad[CgA-E1A] virus was examined on an extended list of cell lines. Cell lines were transduced with the Ad[CgA-E1A] virus, and relative cell viability was measured. Figure 3 shows that after 7 days, Ad[CgA-E1A]

⁵ A. Loskog, personal communication.

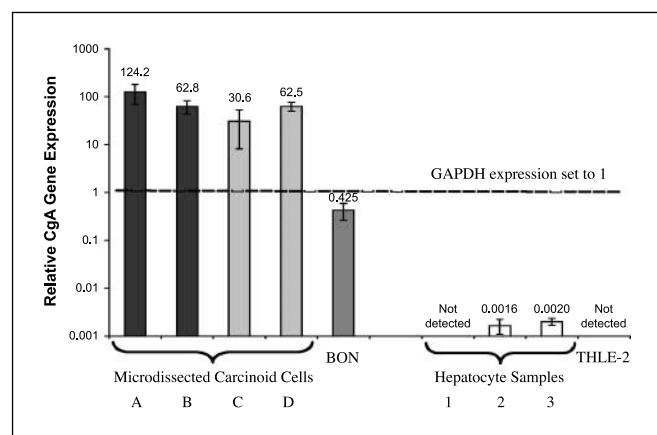


Fig. 6. CgA RNA is highly expressed in midgut carcinoid cells and barely detectable in freshly isolated hepatocytes. Total RNA was isolated from microdissected carcinoid tumor cells from two liver metastases (A and B) and two mesentery metastases (C and D). Total RNA was also isolated from normal freshly isolated hepatocytes from three individuals (1, 2, and 3) and from BON and THLE-2. Thereafter, cDNA was synthesized, and quantitative real-time PCR was done. C_T values were calculated, and the data were evaluated using the $2^{-\Delta\Delta C_T}$ method with GAPDH from each individual sample for normalization. Columns, average values from triplicate samples; bars, SD.

had killed off 100% of BON cells, 80% of SH-SY-5Y, and 75% of SK-N-DZ cells, whereas it had no lytic activity in 1064SK, T24, mel397, or T47D cells. Ad[CMV-E1A] possessed efficient killing of all cell lines examined, whereas Ad[Mock] did not.

Ad[CgA-E1A] suppresses BON carcinoid tumor growth in nude mice. We next went on to examine the efficacy of Ad[CgA-E1A] to control tumor growth *in vivo*. To detect viable tumor cells through *in vivo* bioluminescence imaging, we produced a luciferase and EGFP-expressing line of BON cells, BON(luc/GFP), through retrovirus transformation and cell sorting. These tumor cells were then injected s.c. in nude mice. After 10 days, the established tumors were treated with intratumoral injections of Ad[CgA-E1A] or PBS. The tumors were treated again on days 12, 14, and 17. Tumor growth was monitored through *in vivo* bioluminescence imaging and caliper measurements. An example of *in vivo* luciferase activity detection of BON tumors in two mice treated with Ad[CgA-E1A] and PBS, respectively, is presented in Fig. 4A. The growth of Ad[CgA-E1A]-treated tumor was arrested, whereas the PBS-treated tumor continued to grow as indicated by increased luciferase activity. Tumor sizes for individual mice in the treatment groups are presented in Fig. 4B. We found that four out of five PBS-treated tumors continued to grow rapidly, whereas one out of five grew somewhat slower. On the contrary, the growth of Ad[CgA-E1A]-treated tumors was arrested up to day 40, and in some mice, tumor regression was observed. At day 19, 2 days after the last treatment, there was a statistically significant difference in tumor size between the Ad[CgA-E1A]-treated and PBS-treated mice (Mann-Whitney *U* test, $\alpha = 0.05$; $P = 0.03$). The survival of BON(luc/GFP)-grafted mice is presented in Fig. 4C. There is a significant prolonged survival for Ad[CgA-E1A]-treated mice compared with PBS-treated control mice (Kaplan-Meier log-rank test, $\alpha = 0.05$; $P = 0.004$).

The experiment was stopped on day 61 when the effects of the Ad[CgA-E1A] treatment had diminished. The tumors were excised and examined for evidence of Ad[CgA-E1A] virus

activity by quantitative real-time PCR. Figure 4D shows that E1A mRNA expression was still detectable in tumor cells at day 61, although the tumors were growing in size.

CgA expression in carcinoid metastases and normal hepatocytes: implications for Ad[CgA-E1A] therapy. If Ad[CgA-E1A] is to be used for the treatment of midgut carcinoid liver metastases, it is important that the activity of the virus is low in normal hepatocytes. To investigate this, we isolated hepatocytes from patients undergoing liver surgery. None of the patients had been diagnosed with carcinoid tumors. First, CgA protein expression levels were examined by immunofluorescence microscopy analysis. No CgA protein was detected in normal hepatocytes (Fig. 5, left), whereas BON cells used as a positive control showed granular staining (Fig. 5, right). Next, we extracted total RNA from freshly isolated normal hepatocytes from three individuals (samples 1, 2, and 3) and from the transformed normal liver cell line THLE-2. We also extracted total RNA from BON cells and from laser-assisted microdissected midgut carcinoid tumor cells from two separate liver metastases (A and B) and two separate mesentery metastases (C and D). Quantitative real-time PCR analysis showed that CgA gene expression level is very high in all carcinoid metastases (Fig. 6). The C_T value analysis indicates that the expression level of CgA is at least 15,000 times higher (30.6/0.0020) in microdissected carcinoid tumor cells than in normal

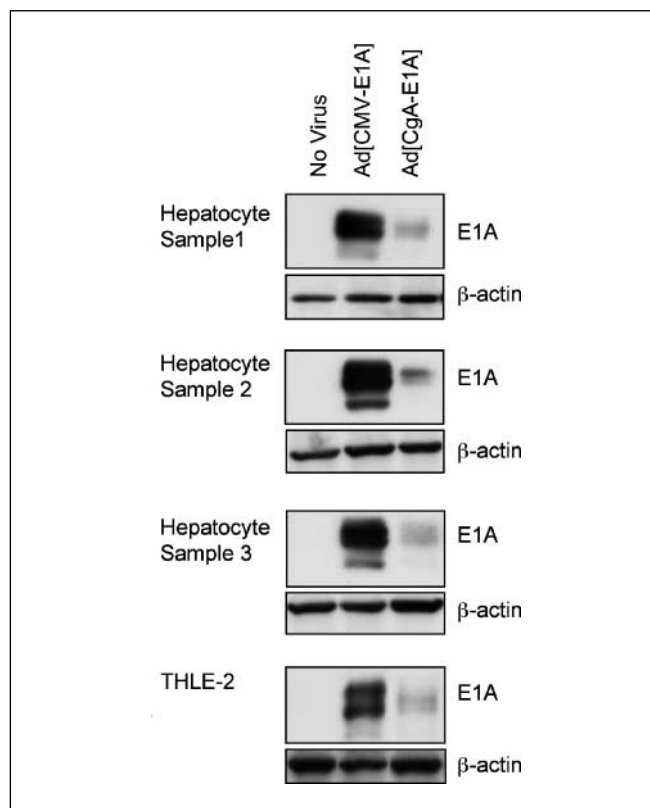


Fig. 7. Ad[CgA-E1A] provides weak E1A expression in hepatocytes. Freshly isolated hepatocytes from three individuals (1, 2, and 3) were cultured overnight, washed, and transduced with Ad[CgA-E1A] and Ad[CMV-E1A]. THLE-2 cells were also transduced with Ad[CgA-E1A] and Ad[CMV-E1A]. Total protein lysates were prepared after 48 h, and 40 μ g samples were resolved by SDS-PAGE. E1A was detected by Western blotting using anti-E1A antibody. Anti- β -actin antibody was used as a control of protein loading.

hepatocytes, when all samples were expressed in relation to the housekeeping gene GAPDH. Furthermore, the expression level of CgA is substantially higher in microdissected carcinoid tumor cells than in BON cells. The transformed liver cell line THLE-2 and one of the hepatocyte samples did not express detectable levels of CgA.

We next transduced freshly isolated hepatocytes (samples 1, 2, and 3) with Ad[CgA-E1A] and Ad[CMV-E1A] and analyzed E1A expression by Western blot. As expected, E1A was strongly expressed in Ad[CMV-E1A]-transduced hepatocytes (Fig. 7). However, Ad[CgA-E1A]-transduced hepatocytes and THLE-2 also showed a faint expression of E1A (Fig. 7). This finding was somewhat surprising when considering that we were unable to detect CgA protein expression (Fig. 5) and were barely able to detect CgA mRNA expression (Fig. 6) in the normal freshly isolated hepatocytes.

Discussion

Because CgA gene expression is restricted to cells of neuroendocrine origin, the CgA promoter may theoretically be used to specifically restrict the expression of therapeutic transgenes to such cells. We show that the CgA promoter can be used to restrict the replication of an oncolytic adenovirus to cells with neuroendocrine features, including BON, which are often used as a model system for carcinoid tumors (35–37). Schipper et al. had previously shown that the CgA promoter can be used to control expression of the human sodium iodine symporter (NIS), an integral plasma membrane glycoprotein normally expressed in thyroid follicular cells (38). The biological function of NIS is to mediate active transport of iodine, a crucial component for thyroid hormone biosynthesis. The transporting ability of NIS is used in radioiodine therapy of thyroid carcinoma. Oncolytic adenoviruses are potent agents for cancer therapy that may be armed with additional therapeutic genes. Therefore, incorporation of NIS in the genome of Ad[CgA-E1A] might be an interesting approach to combine virotherapy and radiotherapy of carcinoid metastases.

Our animal studies clearly show that the Ad[CgA-E1A] virus has oncolytic activity *in vivo* when it is injected into established BON(luc/GFP) tumors. BON(luc/GFP) xenografts are fast-growing tumors, and mice treated with PBS had to be sacrificed within 20 to 40 days. Intratumoral treatment with Ad[CgA-E1A] virus clearly changed the picture with delayed tumor growth, and all treated mice were still alive after 55 days. However, at this point of time, the tumors had started to grow back despite the fact that virus replication could still be detected in excised tumor cells. The reason may be attributed to the fast growth kinetics of BON xenografts in combination with partial clearance of virus by the innate immune system. Carcinoid metastases normally have a slow growth kinetics, which would favor the use of an oncolytic adenovirus, such as Ad[CgA-E1A], that is not dependent on host cell division for replication and lytic activity. Taken together, our data strongly suggest that the

Ad[CgA-E1A] virus could be highly active in human metastatic carcinoid tumors *in situ*.

To use an oncolytic virus for therapy of carcinoid metastases to the liver, it is important that normal liver cells do not support virus replication to minimize side effects of such treatment. The Ad[CgA-E1A] virus could, for example, have some activity in the neuroendocrine compartment of the liver (39). We show that the endogenous CgA gene is highly transcribed in microdissected midgut carcinoid tumor cells both from mesentery and liver metastases. We also show that isolated hepatocytes do not express CgA, which suggests that a therapeutic gene controlled by the CgA promoter would not be expressed in hepatocytes. In contrast, when the same pool of hepatocytes was transduced with Ad[CgA-E1A], we could detect a weak E1A protein expression. The reason behind this weak expression is not known at this time. However, the epigenetic status of the CgA promoter in the genome of hepatocytes may help repress endogenous CgA gene expression, whereas the adenoviral genome is accessible for transcription soon after infection. One possibility is that essential transcription factors that are blocked from binding the genomic CgA promoter in hepatocytes, possibly due to DNA methylation and/or histone deacetylation, may bind to the CgA promoter region in the adenoviral vector and confer weak transcriptional activity. Alternatively, adenoviral backbone sequences may be responsible for read-through transcriptional activity. It is known that adenoviral vector sequences can potentially interfere with the specificity and/or activity of a heterologous promoter (40, 41). Interference may come from the virus origin of replication, i.e., the left inverted terminal repeat (LITR). It may also come from the E1A enhancer, which overlaps with the virus encapsidation sequence needed for virus assembly. As we have shown in the past, the H19 insulator can be used to block or reduce this unwanted transcriptional activity and protect a prostate-specific promoter sequence driving E1A expression (6).⁴ We will look into modifying the CgA-E1A expression cassette in the adenoviral vector background to see if we can reduce the weak unwanted E1A expression in hepatocytes while maintaining the high E1A expression in carcinoid cells. However, it is important to stress that E1A expression from Ad[CgA-E1A] is far weaker in transduced hepatocytes than E1A expression from Ad[CMV-E1A], and that this opens up a therapeutic window. Taken together, our data suggest that the oncolytic Ad[CgA-E1A] virus could be used to treat metastatic carcinoid metastases to the liver either through intratumoral injections or intrahepatic injections possibly in conjunction with standard therapy for these malignancies.

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A Novel Chromogranin-A Promoter-Driven Oncolytic Adenovirus for Midgut Carcinoid Therapy

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